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(71) Applicant *(for all designated States except US)*: **ELI LILLY AND COMPANY** [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **BRADER, Mark, Laurence** [NZ/US]; 5858 Forest Lane, Indianapolis, IN 46220 (US).

(74) Agents: **WALSH, Andrea, C.** et al.; Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285 (US).

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(54) Title: PROTAMINE-FREE INSOLUBLE ACYLATED INSULIN COMPOSITIONS

(57) Abstract: The present invention relates to ultralente-like crystals, comprised of insulin derivatives or insulin analog derivatives, and optionally, underivatized insulin or underivatized insulin analogs. Insoluble compositions comprising the ultralente-like crystals are suitable for both parenteral and non-parenteral delivery for treating hyperglycemia and diabetes.

PROTAMINE-FREE INSOLUBLE ACYLATED INSULIN COMPOSITIONS

Cross Reference

This application claims the benefit of U.S. Provisional Application No. 60/141,435 filed on June 29, 1999, said application of which is entirely incorporated herein by reference.

Background of the Invention

1. Field of the Invention. This invention is in the field of human medicine. More particularly, this invention is in the field of pharmaceutical treatment of the diseases of diabetes and hyperglycemia.

2. Description of Related Art. It has long been a goal of insulin therapy to mimic the pattern of endogenous insulin secretion in normal individuals. The daily physiological demand for insulin fluctuates and can be separated into two phases: (a) the absorptive phase requiring a pulse of insulin to dispose of the meal-related blood glucose surge, and (b) the post-absorptive phase requiring a sustained delivery of insulin to regulate hepatic glucose output for maintaining optimal fasting blood glucose.

Accordingly, effective therapy for people with diabetes generally involves the combined use of two types of exogenous insulin formulations: a rapid acting meal time insulin provided by bolus injections and a long-acting, so-called, basal insulin, administered by injection once or twice daily to control blood glucose levels between meals. An ideal basal insulin will provide an extended and "flat" time action - that is, it will control blood glucose levels for at least 12 hours, and preferably for 24 hours or more,

without significant risk of hypoglycemia. Furthermore, an ideal basal insulin should be mixable with a soluble meal-time insulin, and should not cause irritation or reaction at the site of administration.

As is well understood by those skilled in this art, long-acting insulin formulations have been obtained by formulating normal insulin as microcrystalline suspensions for subcutaneous injection. Examples of commercial insulin preparations used for basal insulin therapy include NPH (Neutral Protamine Hagedorn) insulin, protamine zinc insulin (PZI), and ultralente (UL). These formulations are suspension formulations whereby prolonged insulin activity is achieved by the slow dissolution of solid insulin particles at the subcutaneous site resulting in sustained insulin absorption into the bloodstream. Dissolution of the solid insulin particles at the subcutaneous site is thus the rate-controlling step in determining the pharmacodynamics and pharmacokinetics. The therapeutic characteristics of insulin suspension formulations critical to their efficacy include; the time of onset of insulin activity, the duration of insulin effect, the time and magnitude of maximal effect (i.e. peak), and the overall pharmacokinetic profile. These properties are characteristic for each type of suspension formulation and are determined by the chemical and physical nature of the solid insulin particles of the suspension.

The solid insulin particles of NPH and PZI formulations incorporate protamine which is essential to stabilizing these particular formulations. The term "protamine" refers to a mixture of strongly basic proteins obtained from fish sperm. In contrast, the solid insulin particles of the ultralente formulation do not contain protamine. Ultralente insulin is a microcrystalline complex of insulin and zinc formulated in an aqueous diluent

containing methylparaben, sodium acetate, and sodium chloride. One advantage of the ultralente formulation is the absence of protamine which can cause allergic reactions and injection site inflammation [Galloway J. and deShazo, R., Diabetes Mellitus: Theory and Practice, 25:519-538, ed. 3, Medical Examination Publishing Co. Inc. New Hyde Park, NY, (1983)]. The danger of allergy to protamine lies in the sensitization of patients, by treatment with protamine-containing insulin formulations, for later exposure of such patients to protamine given after cardiac surgery to neutralize the anticoagulant effects of heparin which may result in severe anaphylactic reaction [Galloway J., Diabetes Care, 3:615-622 (1980)].

Ultralente insulin is currently available commercially incorporating recombinant human insulin and was formerly available commercially incorporating pork insulin, beef insulin, or mixtures thereof. The availability of recombinant human insulin in the 1980s resulted in the human ultralente product superceding the animal ultralente products and the latter ceased to be commercially available. The advantage of human ultralente is that its manufacture does not rely on a source of animal pancreases, and the immunogenicity of the human insulin amino acid sequence is less than that of pork insulin and substantially less than that of beef insulin (Ottesen J. et al. Diabetologia (1994) 37:1178-1185). Immunogenicity results in the generation of antibodies to insulin which delay the effect of regular insulin administered to control meal glycemia. Immunogenicity has been recognized as a particular problem with beef insulin suspension formulations [Galloway J. & Chance R. Horm. Met. Res. 26:591-598 (1994)].

Human ultralente, while lacking in immunogenicity, provides only intermediate time action that is not suitably

flat for effective basal insulin therapy. A single daily injection of human ultralente does not provide adequate basal glycemic control and, due to its substantial pharmacokinetic peak, can result in undesirably high levels of insulin in the blood which may cause life-threatening hypoglycemia.

It has been recognized that the pharmacodynamics of beef ultralente are significantly different to those of human ultralente. The pharmacokinetics of beef ultralente are significantly longer and flatter than those of human ultralente, and therefore, is the only long-acting insulin considered to have an ideal basal profile, lacking an onset peak and providing sustained blood insulin levels for more than 24 hours [Galloway J. and Chance R. Horm. Met. Res. 26:591-598 (1994)]. While the pharmacodynamics of beef insulin ultralente represents a near-ideal basal insulin profile, it relies on a supply of pancreases from animal sources and suffers the disadvantage of immunogenicity of the beef insulin amino acid sequence.

It is thus an object of the present invention to provide an insulin suspension formulation that offers the pharmacokinetic profile of beef ultralente, but does not rely on the availability of animal sourced insulin, does not possess the immunogenicity of the beef insulin sequence, and does not incorporate protamine. It is a further object of the present invention to provide filterable crystals of acylated insulins and filterable crystals of acylated insulin and insulin mixture compositions.

For pulmonary administration solid insulin preparations that do not require phenolic preservatives or protamine as stabilizing agents are preferred since protamine and phenolic preservatives are likely to act as irritants in the lung and are, therefore, undesirable in

insulin preparations for inhalation. It is a further object of the present invention to provide solid acylated insulin compositions and solid mixture compositions of acylated insulins and insulin that do not contain phenolic preservatives and do not contain protamine that may be used as pulmonary hypoglycemic agents.

There have been attempts to address the perceived inadequacies of known insulin suspensions. Fatty acid-acylated insulins have been investigated for basal control of blood glucose [Havelund, S., *et al.*, WIPO publication WO95/07931, 23 March 1995]. Their extended time action is caused by binding of the fatty acyl portion of these molecules to serum albumin. The fatty acyl chain lengths of these molecules is such as to take advantage of the fatty acid binding capability of serum albumin. The fatty acid chains used in fatty acid-acylated insulins are typically longer than about ten carbon atoms, and chain lengths of fourteen and sixteen carbon atoms are optimal for binding to serum albumin and extending time action.

Unlike ultralente insulin, which is insoluble, the aforementioned fatty acid-acylated insulins are soluble at the usual therapeutic concentrations of insulin. However, the time action of these preparations may not be sufficiently long enough, or flat enough, to provide ideal basal control, and they are less potent than insulin, thereby requiring administration of greater amounts of the drug agent [Radziuk, J., et al., Diabetologia 41:116-120, 489-490 (1998)].

Thus, there remains a need to identify insulin preparations that have flatter and longer time action than NPH insulin and that do not pose risk of irritation or reaction at the site of administration. It was discovered quite surprisingly that ultralente-like compositions that

include an acylated insulin and zinc can be prepared and that ultralente-like compositions that include a mixture of an acylated insulin and insulin and zinc can be prepared. In addition to the properties mentioned above, the insoluble compositions are expected to provide flexibility of control over the duration and shape of the glucodynamic response profile. They are thought to function as controlled release compositions, wherein, the release rate is controlled by the proportion and nature of the derivatized protein. Other aspects of this invention that relate to the preparation, formulation, and use of such compositions will be discussed herein.

There are no examples known to me of compositions of acylated insulins and mixtures of acylated insulins with insulin, as those terms are to be understood in the context of the present disclosure.

The closest art relates to crystals comprised of proinsulin and insulin [Steiner, D. F., Nature *243*:528-530 (1973); Low, B. W., et al., Nature *248*:339-340 (1974)] and to crystals comprised of a insulin or an insulin analog having approximately the same isoelectric point as insulin and an insulin analog having additional basic amino acids [Dörschug, M., *et al.*, U.S. Patent No. 5,028,587, issued 2 July 1991].

Steiner produced crystals comprised of proinsulin and insulin with mole ratios of about 1:11, 1:5, 1:2, and 1:1, respectively (i.e., 0.5, 1, 2, and 3 moles of proinsulin per 6 moles total insulin and proinsulin) in 0.095 M sodium citrate, pH 6.0, 0.03 M NaCl, 0.012 M ZnCl2, and 16% acetone. The proportion of proinsulin greatly affected the rate of crystallization. The crystals differed greatly from those of pure insulin under the same conditions, and were characterized as rhombohedral crystals

with rounded borders. There was great variability within and between preparations. The utility ascribed to crystallizing proinsulin and insulin was that it facilitated isolating small amounts of proinsulin and related structures from pancreatic extracts. The author speculated that crystallization might occur between precursor and product peptides, and among other closely related proteins.

Low, B. W., *et al.* produced very large crystals comprised of equimolar proportions of beef or pork insulin and their respective proinsulins, wherein the proinsulin and insulin were formed into homogenous hexamers prior to crystallization. Analysis by X-ray crystallography and quantitative electrophoresis supported a conclusion that the unit cell in the crystals was formed of twelve insulin hexamers and twelve proinsulin hexamers. It was specifically stated that no studies were known to suggest that insulin and proinsulin form mixed dimers and hexamers in solution.

Dörschug, M., *et al.* disclosed crystals comprised of insulin, des(PheB1) insulin, des(ThrB30) human insulin, or des(AlaB30) beef insulin, and at least one insulin having a basic modification at the C-terminal end of the B chain ("modified insulin"). Such modified insulins are disclosed, for example, in European Patent Application No. 132,769. Globin or protamine sulfate were stated to be auxiliary compounds that could be used in the crystal preparations. There are no examples of the use of protamine, nor any suggestion that the inventors appreciated the effect of adding such compounds. Furthermore, the modified insulins used in Dörschug, *et al.* are different than the derivatives used in the present invention.

The present invention is based on the surprising discovery that it is possible to prepare ultralente-like

crystals with acylated insulins as well as ultralente-like co-crystals containing a mixture of acylated insulin and insulin. The ultralente-like crystals described in this invention were prepared and characterized by HPLC.

Summary Of The Invention

The present invention provides microcrystalline compositions of acylated insulins and microcrystalline mixture compositions of acylated insulin and insulin to provide therapeutic basal insulin activity without the use of insulin from animal sources, avoiding the immunogenicity of beef insulin, and without the use of protamine. The present invention also provides easily filterable microcrystalline compositions of acylated insulins and microcrystalline compositions of acylated insulin and insulin mixtures.

Accordingly, in its broadest aspect, the present invention provides insoluble compositions comprising a derivatized protein selected from the group consisting of acylated insulin derivatives and acylated insulin analog derivatives, a protein selected from the group consisting of insulin and insulin analogs, and a divalent metal cation. The insoluble compositions of the present invention are in the form of microcrystals, or in the form of mixtures of microcrystals and amorphous precipitates. These insoluble compositions are useful for treating diabetes and hyperglycemia, and provide the advantages of having flatter and longer time action than NPH insulin. Furthermore, by varying the ratio between protein and derivatized protein, the extent of protraction of the time action can be finely controlled over a very great range of time-action, from that nearly the same as NPH insulin to much greater than that of NPH insulin.

The present invention is distinct from previous fatty acid-acylated insulin technology in that the extension of time action of the present invention does not rely necessarily on albumin-binding, though albumin binding may further protract the time action of certain of the compositions of the present invention.

The microcrystals of the present invention are useful for treating diabetes and for controlling blood glucose in a patient in need thereof.

The invention provides aqueous suspension formulations comprising an insoluble composition and an aqueous solvent. One such aqueous suspension formulation is comprised of a microcrystalline composition of the present invention and an aqueous solvent. The formulations of the present invention have superior pharmacodynamics compared with human insulin NPH, and their time-action can be purposefully selected over a wide range, from just slightly extended compared with human insulin NPH to very greatly extended compared with human insulin NPH.

The invention provides a method of treating diabetes or hyperglycemia comprising, administering to a patient in need thereof a sufficient quantity of an insoluble composition of the present invention to regulate blood glucose levels in the patient.

Description Of The Invention

As used herein, the term "co-crystal" means a microcrystal of the present invention.

The term "insoluble composition" refers to matter in either a microcrystalline state or in an amorphous precipitate state, or both. The presence of microcrystals or amorphous precipitate can be ascertained by visual and

microscopic examination. Solubility depends on solvent, and a particular composition may be insoluble in one solvent, but soluble in another.

The term "microcrystal" means a solid that is comprised primarily of matter in a crystalline state, wherein the individual crystals are predominantly of a single crystallographic composition and are of a microscopic size, typically of longest dimension within the range 1 micron to 100 microns. The term "microcrystalline" refers to the state of being a microcrystal.

The term "amorphous precipitate" refers to insoluble protein or derivatized protein that is not crystalline in form. The person of ordinary skill can distinguish crystals from amorphous precipitate. The amorphous precipitates of the present invention have advantageous pharmacological properties in their own right, and also are intermediates in the formation of the microcrystals of the present invention.

The term "protein" may have its common meaning, that is, a polymer of amino acids. The term "protein," as used herein, also has a narrower meaning, that is, a protein selected from the group consisting of insulin and insulin analogs. The term "un-derivatized protein" also refers to a protein selected from the group consisting of insulin and insulin analogs.

As used in the claims, and elsewhere as the context dictates, the term "total protein" refers to the combined amount of protein (insulin or insulin analog) and derivatized protein (derivatized insulin or a derivatized insulin analog).

The term "derivatized protein" refers to a protein selected from the group consisting of derivatized insulin and derivatized insulin analogs that is derivatized by a

functional group such that the derivatized protein is less soluble in an aqueous solvent than is the un-derivatized protein. Many examples of such derivatized proteins are known in the art, and the determination of solubility of proteins and derivatized proteins is well known to the skilled person. Examples of derivatized insulin and insulin analogs include benzoyl, *p*-tolyl-sulfonamide carbonyl, and indolyl derivatives of insulin and insulin analogs [Havelund, S., *et al.*, WO95/07931, published 23 March 1995]; alkyloxycarbonyl derivatives of insulin [Geiger, R., *et al.*, U.S. Patent No. 3,684,791, issued 15 August 1972; Brandenberg, D., *et al.*, U.S. 3,907,763, issued 23 September 1975]; aryloxycarbonyl derivatives of insulin [Brandenberg, D., *et al.*, U.S. 3,907,763, issued 23 September 1975]; alkylcarbamyl derivatives [Smyth, D. G., U.S. Patent No. 3,864,325, issued 4 February 1975; Lindsay, D. G., *et al.*, U.S. Patent No. 3,950,517, issued 13 April 1976]; carbamyl, O-acetyl derivatives of insulin [Smyth, D. G., U.S. Patent No. 3,864,325 issued 4 February 1975]; cross-linked, alkyl dicarboxyl derivatives [Brandenberg, D., *et al.*, U.S. Patent No. 3,907,763, issued 23 September 1975]; N-carbamyl, O-acetylated insulin derivatives [Smyth, D. G., U.S. Patent No. 3,868,356, issued 25 February 1975]; various O-alkyl esters [Markussen, J., U.S. Patent No. 4,343,898, issued 10 August 1982; Morihara, K., *et al.*, U.S. Patent No. 4,400,465, issued 23 August 1983; Morihara, K., *et al.*, U.S. Patent No. 4,401,757, issued 30 August 1983; Markussen, J., U.S. Patent No. 4,489,159, issued 18 December 1984; Obermeier, R., *et al.*, U.S. Patent No. 4,601,852, issued 22 July 1986; and Andersen, F. H., *et al.*, U.S. Patent No. 4,601,979, issued 22 July 1986]; alkylamide derivatives of insulin [Balschmidt, P., *et al.*, U.S. Patent No. 5,430,016, issued 4 July 1995]; various other derivatives of insulin

[Lindsay, D. G., U.S. Patent No. 3,869,437, issued 4 March 1975]; and the fatty acid-acylated proteins that are described herein.

The term "acylated protein" as used herein refers to a derivatized protein selected from the group consisting of insulin and insulin analogs that is acylated with an organic acid moiety that is bonded to the protein through an amide bond formed between the acid group of an organic acid compound and an amino group of the protein. In general, the amino group may be the α-amino group of an N-terminal amino acid of the protein, or may be the ε-amino group of a Lys residue of the protein. An acylated protein may be acylated at one or more of the three amino groups that are present in insulin and in most insulin analogs. Mono-acylated proteins are acylated at a single amino group. Di-acylated proteins are acylated at two amino groups. Tri-acylated proteins are acylated at three amino groups. The organic acid compound may be, for example, a fatty acid, an aromatic acid, or any other organic compound having a carboxylic acid group that will form an amide bond with an amino group of a protein, and that will cause the aqueous solubility of the derivatized protein to be lower than the solubility of the un-derivatized protein.

The term "fatty acid-acylated protein" refers to a an acylated protein selected from the group consisting of insulin and insulin analogs that is acylated with a fatty acid that is bonded to the protein through an amide bond formed between the acid group of the fatty acid and an amino group of the protein. In general, the amino group may be the α-amino group of an N-terminal amino acid of the protein, or may be the ε-amino group of a Lys residue of the protein. A fatty acid-acylated protein may be acylated at one or more of the three amino groups that are present in

insulin and in most insulin analogs. Mono-acylated proteins are acylated at a single amino group. Di-acylated proteins are acylated at two amino groups. Tri-acylated proteins are acylated at three amino groups. Fatty acid-acylated insulin is disclosed in a Japanese patent application 1-254,699. *See also,* Hashimoto, M., *et al.*, *Pharmaceutical Research,* 6:171-176 (1989), and Lindsay, D. G., *et al.*, *Biochemical J.* 121:737-745 (1971). Further disclosure of fatty acid-acylated insulins and fatty acylated insulin analogs, and of methods for their synthesis, is found in Baker, J. C., *et al,* U.S. 08/342,931, filed 17 November 1994 and issued as U.S. Patent No. 5,693,609, 2 December 1997; Havelund, S., *et al.*, WO95/07931, published 23 March 1995, and a corresponding U.S. Patent No. 5,750,497, 12 May 1998; and Jonassen, I., *et al.*, WO96/29342, published 26 September 1996.

The term "fatty acid-acylated protein" includes pharmaceutically acceptable salts and complexes of fatty acid-acylated proteins. The term "fatty acid-acylated protein" also includes preparations of acylated proteins wherein the population of acylated protein molecules is homogeneous with respect to the site or sites of acylation. For example, Nε-mono-acylated protein, B1-Nα-mono-acylated protein, A1-Nα-mono-acylated protein, A1,B1-Nα-di-acylated protein, Nε,A1-Nα,di-acylated protein, Nε,B1-Nα,di-acylated protein, and Nε,A1,B1-Nα,tri-acylated protein are all encompassed within the term "fatty acid-acylated protein" for the purpose of the present invention. The term also refers to preparations wherein the population of acylated protein molecules has heterogeneous acylation. In the latter case, the term "fatty acid-acylated protein" includes mixtures of mono-acylated and di-acylated proteins, mixtures of mono-acylated and tri-acylated proteins, mixtures of di-

acylated and tri-acylated proteins, and mixtures of mono-acylated, di-acylated, and tri-acylated proteins.

The term "insulin" as used herein, refers to human insulin, whose amino acid sequence and special structure are well known. Human insulin is comprised of a twenty-one amino acid A-chain and a thirty-amino acid B-chain which are cross-linked by disulfide bonds. A properly cross-linked insulin contains three disulfide bridges: one between position 7 of the A-chain and position 7 of the B-chain, a second between position 20 of the A-chain and position 19 of the B-chain, and a third between positions 6 and 11 of the A-chain.

The term "insulin analog" means proteins that have an A-chain and a B-chain that have substantially the same amino acid sequences as the A-chain and B-chain of human insulin, respectively, but differ from the A-chain and B-chain of human insulin by having one or more amino acid deletions, one or more amino acid replacements, and/or one or more amino acid additions that do not destroy the insulin activity of the insulin analog.

"Animal insulins" are analogs of human insulin, and therefore, are insulin analogs, as defined herein. Four such animal insulins are rabbit, pork, beef, and sheep insulin. The amino acid substitutions that distinguish these animal insulins from human insulin are presented below for the reader's convenience.

	Amino Acid Position			
	A8_	A9_	A10_	B30_
human insulin	Thr	Ser	Ile	Thr
rabbit insulin	Thr	Ser	Ile	Ser
pork insulin	Thr	Ser	Ile	Ala
beef insulin	Ala	Ser	Val	Ala
sheep insulin	Ala	Gly	Val	Ala

Another type of insulin analog, "monomeric insulin analog" is well known in the art. Monomeric insulin analogs are structurally very similar to human insulin, and have activity similar or equal to human insulin, but have one or more amino acid deletions, replacements or additions that tend to disrupt the contacts involved in dimerization and hexamerization which results in their greater tendency to dissociate to less aggregated states. Monomeric insulin analogs are rapid-acting analogs of human insulin, and are disclosed, for example, in Chance, R. E., *et al.*, U.S. patent No. 5,514,646, 7 May 1996; Brems, D. N., *et al. Protein Engineering*, 5:527-533 (1992); Brange, J. J. V., *et al.*, EPO publication No. 214,826, published 18 March 1987; Brange, J. J. V., *et al.*, U.S. Patent No. 5,618,913, 8 April 1997; and Brange, J., *et al., Current Opinion in Structural Biology* 1:934-940 (1991). An example of monomeric insulin analogs is described as human insulin wherein Pro at position B28 is substituted with Asp, Lys, Leu, Val, or Ala, and wherein Lys at position B29 is Lys or is substituted with Pro, and also, AlaB26-human insulin, des (B28-B30)-human insulin, and des (B27)-human insulin. The monomeric insulin analogs employed as derivatives in the present crystals, or employed un-derivatized in the solution phase of suspension formulations, are properly cross-linked at the same positions as is human insulin.

Another group of insulin analogs consists of insulin analogs that have one or more amino acid deletions that do not significantly disrupt the activity of the molecule. This group of insulin analogs is designated herein as "deletion analogs." For example, insulin analogs with deletion of one or more amino acids at positions B1-B3 are active. Likewise, insulin analogs with deletion of one

or more amino acids at positions B28-B30 are active. Examples of "deletion analogs" include des(B30)-human insulin, desPhe(B1)-human insulin, des(B27)-human insulin, des(B28-B30)-human insulin, and des(B1-B3)-human insulin. The deletion analogs employed as derivatives in the present crystals, or employed un-derivatized in the solution phase of suspension formulations, are properly cross-linked at the same positions as is human insulin.

Amidated amino acids, and particularly asparagine residues in insulin, are known to be chemically unstable. Particularly, they are prone to deamidation and various rearrangement reactions under certain conditions that are well known. Therefore, optionally, an insulin analog may be insulin or an insulin analog that has one or more of its amidated residues replaced with other amino acids for the sake of chemical stability. For example, Asn or Gln may be replaced with a non-amidated amino acid. Preferred amino acid replacements for Asn or Gln are Gly, Ser, Thr, Asp or Glu. It is preferred to replace one or more Asn residues. In particular, AsnA18, AsnA21, or AsnB3, or any combination of those residues may be replaced by Gly, Asp, or Glu, for example. Also, GlnA15 or GlnB4, or both, may be replaced by either Asp or Glu. Preferred replacements are Asp at B21, and Asp at B3.

A "pharmaceutically acceptable salt" means a salt formed between any one or more of the charged groups in a protein and any one or more pharmaceutically acceptable, non-toxic cations or anions. Organic and inorganic salts include, for example, those prepared from acids such as hydrochloric, sulfuric, sulfonic, tartaric, fumaric, hydrobromic, glycolic, citric, maleic, phosphoric, succinic, acetic, nitric, benzoic, ascorbic, p-toluenesulfonic, benzenesulfonic, naphthalenesulfonic, propionic, carbonic,

and the like, or for example, ammonium, sodium, potassium, calcium, or magnesium.

The verb "acylate" means to form the amide bond between a fatty acid and an amino group of a protein. A protein is "acylated" when one or more of its amino groups is combined in an amide bond with the acid group of a fatty acid.

The term "fatty acid" means a saturated or unsaturated, straight chain or branched chain fatty acid, having from one to eighteen carbon atoms.

The term "C1 to C18 fatty acid" refers to a saturated, straight chain or branched chain fatty acid having from one to eighteen carbon atoms.

The term "divalent metal cation" refers to the ion or ions that participate to form a complex with a multiplicity of protein molecules. The transition metals, the alkaline metals, and the alkaline earth metals are examples of metals that are known to form complexes with insulin. The transition metals are preferred. Zinc is particularly preferred. Other transition metals that may be pharmaceutically acceptable for complexing with insulin proteins include copper, cobalt, and iron.

The term "complex" has two meanings in the present invention. In the first, the term refers to a complex formed between one or more atoms in the proteins that form the complex and one or more divalent metal cations. The atoms in the proteins serve as electron-donating ligands. The proteins typically form a hexamer complex with divalent transition metal cations.

The term "suspension" refers to a mixture of a liquid phase and a solid phase that consists of insoluble or sparingly soluble particles that are larger than colloidal size. Mixtures of ultralente-like microcrystals and an

aqueous solvent form suspensions. Mixtures of amorphous precipitate and an aqueous solvent also form a suspension. The term "suspension formulation" means a pharmaceutical composition wherein an active agent is present in a solid phase, for example, a microcrystalline solid, an amorphous precipitate, or both, which is finely dispersed in an aqueous solvent. The finely dispersed solid is such that it may be suspended in a fairly uniform manner throughout the aqueous solvent by the action of gently agitating the mixture, thus providing a reasonably uniform suspension from which a dosage volume may be extracted. Examples of commercially available insulin suspension formulations include, for example, NPH, PZI, and ultralente. A small proportion of the solid matter in a microcrystalline suspension formulation may be amorphous. Preferably, the proportion of amorphous material is less than 10%, and most preferably, less than 1% of the solid matter in a microcrystalline suspension. Likewise, a small proportion of the solid matter in an amorphous precipitate suspension may be microcrystalline.

The term "Ultralente-like crystals" refers to crystals of the present invention that are morphologically similar or identical to the ultralente crystals described in Schlichtkrull U.S. Patent 2,799,622, issued July 16, 1957, U.S. Patent 2,819, 999, issued Jan. 14, 1958, and Insulin Crystals, by Schlichtkrull, Ejnar Munksgaard Publishers, Copenhagen (1958). Ultralente-like crystals are comprised of an insulin derivative and optionally insulin or an insulin analog, and zinc.

The crystals of the present invention have rhombohedral morphology or an irregular morphology.

The term "seed crystals" is well known to one of ordinary skill in the art. It refers to a preparation of

insulin-related crystals involving lyophilization as described in Schlichtkrull, U. S. Patent 2,819,999 issued Jan. 14, 1958.

The term "aqueous solvent" refers to a liquid solvent that contains water. An aqueous solvent system may be comprised solely of water, may be comprised of water plus one or more miscible solvents, and may contain solutes. The more commonly used miscible solvents are the short-chain organic alcohols, such as, methanol, ethanol, propanol, short-chain ketones, such as acetone, and polyalcohols, such as glycerol.

An "isotonicity agent" is a compound that is physiologically tolerated and imparts a suitable tonicity to a formulation to prevent the net flow of water across cell membranes that are in contact with an administered formulation. Glycerol, which is also known as glycerin, is commonly used as an isotonicity agent. Other isotonicity agents include salts, *e.g.*, sodium chloride, and monosaccharides, *e.g.*, dextrose and lactose.

The term "preservative" refers to a compound added to a pharmaceutical formulation to act as an anti-microbial agent. A parenteral formulation must meet guidelines for preservative effectiveness to be a commercially viable multi-use product. Among preservatives known in the art as being effective and acceptable in parenteral formulations are benzalkonium chloride, benzethonium, chlorohexidine, phenol, m-cresol, benzyl alcohol, methylparaben, chlorobutanol, o-cresol, p-cresol, chlorocresol, phenylmercuric nitrate, thimerosal, benzoic acid, and various mixtures thereof. *See, e.g.*, Wallhäusser, K.-H., *Develop. Biol. Standard,* 24:9-28 (1974) (S. Krager, Basel).

The term "phenolic preservative" includes the compounds phenol, m-cresol, o-cresol, p-cresol,

chlorocresol, methylparaben, and mixtures thereof. Certain phenolic preservatives, such as phenol and m-cresol, are known to bind to insulin-like molecules and thereby to induce conformational changes that increase either physical or chemical stability, or both [Birnbaum, D. T., *et al.*, *Pharmaceutical. Res.* 14:25-36 (1997); Rahuel-Clermont, S., *et al.*, *Biochemistry* 36:5837-5845 (1997)].

The term "buffer" or "pharmaceutically acceptable buffer" refers to a compound that is known to be safe for use in insulin formulations and that has the effect of controlling the pH of the formulation at the pH desired for the formulation. The pH of the formulations of the present invention is from about 6.0 to about 8.0. Preferably the formulations of the present invention have a pH between about 6.8 and about 7.8. Pharmaceutically acceptable buffers for controlling pH at a moderately acidic pH to a moderately basic pH include such compounds as phosphate, acetate, citrate, arginine, TRIS, and histidine. "TRIS" refers to 2-amino-2-hydroxymethyl-1,3,-propanediol, and to any pharmacologically acceptable salt thereof. The free base and the hydrochloride form are two common forms of TRIS. TRIS is also known in the art as trimethylol aminomethane, tromethamine, and tris(hydroxymethyl)aminomethane. Other buffers that are pharmaceutically acceptable, and that are suitable for controlling pH at the desired level are known to the chemist of ordinary skill.

The term "administer" means to introduce a formulation of the present invention into the body of a patient in need thereof to treat a disease or condition.

The term "treating" refers to the management and care of a patient having diabetes or hyperglycemia, or other condition for which insulin administration is indicated for

the purpose of combating or alleviating symptoms and complications of those conditions. Treating includes administering a formulation of present invention to prevent the onset of the symptoms or complications, alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

The insoluble compositions of the present invention may comprise crystals with rhombohedral morphology or with an irregular morphology, or they may comprise amorphous precipitates.

A preferred group of insulin analogs for preparing derivatized insulin analogs used to form crystals and co-crystals consists of animal insulins, deletion analogs, and pI-shifted analogs. A more preferred group consists of animal insulins and deletion analogs. Deletion analogs are yet more preferred.

Another preferred group of insulin analogs for use in the crystals and co-crystals of the present invention consists of the monomeric insulin analogs. Particularly preferred are those monomeric insulin analogs wherein the amino acid residue at position B28 is Asp, Lys, Leu, Val, or Ala, the amino acid residue at position B29 is Lys or Pro, the amino acid residue at position B10 is His or Asp, the amino acid residue at position B1 is Phe, Asp or deleted alone or in combination with a deletion of the residue at position B2, the amino acid residue at position B30 is Thr, Ala, Ser, or deleted, and the amino acid residue at position B9 is Ser or Asp; provided that either position B28 or B29 is Lys.

Another preferred group of insulin analogs for use in the present invention consists of those wherein the isoelectric point of the insulin analog is between about 7.0 and about 8.0. These analogs are referred to as "pI-shifted

insulin analogs." Examples of pI-shifted insulin analogs include, for example, ArgB31,ArgB32-human insulin, GlyA21,ArgB31,ArgB32-human insulin, ArgA0,ArgB31,ArgB32-human insulin, and ArgA0,GlyA21,ArgB31,ArgB32-human insulin.

Another preferred group of insulin analogs consists of LysB28,ProB29-human insulin (B28 is Lys; B29 is Pro); AspB28-human insulin (B28 is Asp), AspB1-human insulin, ArgB31,ArgB32-human insulin, ArgA0-human insulin, AspB1,GluB13-human insulin, AlaB26-human insulin, GlyA21-human insulin, des(ThrB30)-human insulin, and GlyA21,ArgB31,ArgB32-human insulin.

Especially preferred insulin analogs include LysB28,ProB29-human insulin, des(ThrB30)-human insulin, AspB28-human insulin, and AlaB26-human insulin. Another especially preferred insulin analog is GlyA21, ArgB31, ArgB32-human insulin [Dörschug, M., U. S. Patent No. 5,656,722, 12 August 1997]. The most preferred insulin analog is LysB28,ProB29-human insulin.

The preferred derivatized proteins are acylated proteins, and the preferred acylated proteins for the microcrystals and formulations of the present invention are fatty acid-acylated insulin and fatty acid-acylated insulin analogs. Fatty acid-acylated human insulin is highly preferred. Fatty acid-acylated insulin analogs are also highly preferred.

The particular group used to derivatize insulin or an insulin analog (collectively, protein) may be any chemical moiety that does not significantly reduce the biological activity of the protein, is not toxic when bonded to the protein, and most importantly, reduces the aqueous solubility, raises the lipophilicity, or decreases the solubility of zinc complexes of the derivatized protein.

One preferred group of acylating moieties consists of fatty acids that are straight chain and saturated. This group consists of methanoic acid (C1), ethanoic acid (C2), propanoic acid (C3), n-butanoic acid (C4), n-pentanoic acid (C5), n-hexanoic acid (C6), n-heptanoic acid (C7), n-octanoic acid (C8), n-nonanoic acid (C9), n-decanoic acid (C10), n-undecanoic acid (C11), n-dodecanoic acid (C12), n-tridecanoic acid (C13), n-tetradecanoic acid (C14), n-pentadecanoic acid (C15), n-hexadecanoic acid (C16), n-heptadecanoic acid (C17), and n-octadecanoic acid (C18). Adjectival forms are formyl (C1), acetyl (C2), propionyl (C3), butyryl (C4), pentanoyl (C5), hexanoyl (C6), heptanoyl (C7), octanoyl (C8), nonanoyl (C9), decanoyl (C10), undecanoyl (C11), dodecanoyl (C12), tridecanoyl (C13), tetradecanoyl (C14) or myristoyl, pentadecanoyl (C15), hexadecanoyl (C16) or palmitic, heptadecanoyl (C17), and octadecanoyl (C18) or stearic.

A preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having an even number of carbon atoms - that is, C2, C4, C6, C8, C10, C12, C14, C16, and C18 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having an odd number of carbon atoms - that is, C1, C3, C5, C7, C9, C11, C13, C15, and C17 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having more than 5 carbon atoms - that is, C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, C16, C17, and C18 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having less than 9 carbon atoms - that is, C1, C2, C3, C4, C5, C6, C7, and C8 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having between 6 and 8 carbon atoms - that is, C6, C7, and C8, saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having more than between 4 and 6 carbon atoms - that is, C4, C5, and C6, saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having more than between 2 and 4 carbon atoms - that is, C2, C3, and C4, saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having less than 6 carbon atoms - that is, C1, C2, C3, C4, and C5 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having less than 4 carbon atoms - that is, C1, C2, and C3 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having more

than 9 carbon atoms - that is, C10, C11, C12, C13, C14, C15, C16, C17, and C18 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having an even number of carbon atoms and more than 9 carbon atoms - that is, C10, C12, C14, C16, and C18 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having 12, 14, or 16 carbon atoms, that is, C12, C14, and C16 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having 14 or 16 carbon atoms, that is, C14 and C16 saturated fatty acids. Fatty acids with 14 carbons are particularly preferred. Fatty acids with 16 carbons are also particularly preferred.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of saturated fatty acids having between 4 and 10 carbon atoms, that is C4, C5, C6, C7, C8, C9, and C10 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of saturated fatty acids having an even number of carbon atoms between 4 and 10 carbon atoms, that is C4, C6, C8, and C10 saturated fatty acids.

Another preferred group of fatty acids for forming the fatty acid-acylated proteins used in the microcrystals of the present invention consists of fatty acids having 6,

8, or 10 carbon atoms. Fatty acids with 6 carbons are particularly preferred. Fatty acids with 8 carbons are also particularly preferred. Fatty acids with 10 carbons are particularly preferred.

The skilled person will appreciate that narrower preferred groups are made by combining the preferred groups of fatty acids described above.

Another preferred group of acylating moieties consists of saturated fatty acids that are branched. A branched fatty acid has at least two branches. The length of a "branch" of a branched fatty acid may be described by the number of carbon atoms in the branch, beginning with the acid carbon. For example, the branched fatty acid 3-ethyl-5-methylhexanoic acid has three branches that are five, six, and six carbons in length. In this case, the "longest" branch is six carbons. As another example, 2,3,4,5-tetraethyloctanoic acid has five branches that are 4, 5, 6, 7, and 8 carbons long. The "longest" branch is eight carbons. A preferred group of branched fatty acids are those having from three to ten carbon atoms in the longest branch.

A representative number of such branched, saturated fatty acids will be mentioned to assure the reader's comprehension of the range of such fatty acids that may be used as acylating moieties of the proteins in the present invention:

4 Carbons:2-methyl-propioinic acid,

5 Carbons:2-methyl-butyric acid, 3-methyl-butyric acid, 2,2-dimethyl-propionic acid,

6 Carbons:2-methyl-pentanoic acid, 3-methyl-pentanoic acid, 4-methyl-pentanoic acid, 2,2-dimethyl-butyric acid, 2,3-dimethyl-butyric acid, 3,3-dimethyl-butyric acid, 2-ethyl-butyric acid,

7 Carbons:2-methyl-hexanoic acid, 5-methyl-hexanoic acid, 2,2-dimethyl-pentanoic acid, 2,4-dimethyl-pentanoic acid, 2-ethyl-3-methyl-butyric acid, 2-ethyl-pentanoic acid, 3-ethyl-pentanoic acid, 2,2-dimethyl-3-methyl-butyric acid,

8 Carbons:2-methyl-heptanoic acid, 3-methyl-heptanoic acid, 4-methyl-heptanoic acid, 5-methyl-heptanoic acid, 6-methyl-heptanoic acid, 2,2-dimethyl-hexanoic acid, 2,3-dimethyl-hexanoic acid, 2,4-dimethyl-hexanoic acid, 2,5-dimethyl-hexanoic acid, 3,3,-dimethyl-hexanoic acid, 3,4-dimethyl-hexanoic acid, 3,5-dimethyl-hexanoic acid, 4,4-dimethyl-hexanoic acid, 2-ethyl-hexanoic acid, 3-ethyl-hexanoic acid, 4-ethyl-hexanoic acid, 2-propyl-pentanoic acid, 2-ethyl-hexanoic acid, 3-ethyl-hexanoic acid, 4-ethyl-hexanoic acid, 2-(1-propyl)pentanoic acid, 2-(2-propyl)pentanoic acid, 2,2-diethyl-butyric acid, 2,3,4-trimethyl-pentanoic acid,

9 Carbons:2-methyl-octanoic acid, 4-methyl-octanoic acid, 7-methyl-octanoic acid, 2,2-dimethyl-heptanoic acid, 2,6-dimethyl-heptanoic acid, 2-ethyl-2-methyl-hexanoic acid, 3-ethyl-5-methyl-hexanoic acid, 3-(1-propyl)-hexanoic acid, 2-(2-butyl)-pentanoic acid, 2-(2-(2-methylpropyl))pentanoic acid,

10 Carbons:2-methyl-nonanoic acid, 8-methyl-nonanoic acid, 6-ethyl-octanoic acid, 4-(1-propyl)-heptanoic acid, 5-(2-propyl)-heptanoic acid,

11 Carbons:3-methyl-undecanoic acid,

12 Carbons:2-pentyl-heptanoic acid, 2,3,4,5,6-pentamethyl-heptanoic acid, 2,6-diethyl-octanoic acid,

14 Carbons:2-hexyl-octanoic acid, 2,3,4,5,6,7-hexamethyl-octanoic acid, 3,3-diethyl-4,4-diethyl-hexanoic acid,

16 Carbons:2-heptyl-nonanoic acid, 2,3,4,5-tetraethyl-octanoic acid,

18 Carbons:2-octyl-decanoic acid, and 2-(1-propyl)-3-(1-propyl)-4,5-diethyl-6-methyl-heptanoic acid.

Yet another preferred group of acylating moieties consists of cyclic alkyl acids having from 5 to 24 carbon atoms, wherein the cyclic alkyl moiety, or moieties, have 5 to 7 carbon atoms. A representative number of such cyclic alkyl acids will be mentioned to assure the reader's comprehension of the range of such acids that may be used as acylating moieties of the proteins in the present invention: cyclopentyl-formic acid, cyclohexyl-formic acid, 1-cyclopentyl-acetic acid, 2-cyclohexyl-acetic acid, 1,2-dicyclopentyl-acetic acid, and the like.

A preferred group of derivatized proteins consists of mono-acylated proteins. Mono-acylation at the ε-amino group is most preferred. For insulin, mono-acylation at LysB29 is preferred. Similarly, for certain insulin analogs, such as, LysB28,ProB29-human insulin analog, mono-acylation at the ε-amino group of LysB28 is most preferred. Mono-acylation at the α-amino group of the B-chain (B1) is also preferred. Mono-acylation at the α-amino group of the A-chain (A1) is also preferred.

Another group of acylated proteins consists of di-acylated proteins. The di-acylation may be, for example, at the ε-amino group of Lys and at the α-amino group of the B-chain, or may be at the ε-amino group of Lys and at the α-amino group of the A-chain, or may be at the α-amino group the A-chain and at the α-amino group of the B-chain.

Another group of acylated proteins consists of tri-acylated proteins. Tri-acylated proteins are those that are acylated at the ε-amino group of Lys, at the α-amino

group of the B-chain, and at the α-amino group of the A-chain.

Aqueous compositions containing water as the major solvent are preferred. Aqueous suspensions wherein water is the solvent are highly preferred.

The compositions of the present invention further comprises a divalent metal cation. The transition metals are preferred. Zinc is particularly preferred. Other transition metals that may be pharmaceutically acceptable for complexing with insulin proteins include copper, cobalt and iron.

The primary role of divalent metal cations such as zinc in the present invention is to facilitate formation of hexamers of the protein. Zinc facilitates the formation of hexamers of insulin, animal insulins and insulin analogs. Zinc likewise promotes the formation of hexamers of derivatized insulin, insulin analogs and animal insulins.

The composition of the present invention may further comprise a buffer, preferably a pharmaceutically acceptable buffer. Preferred buffers include TRIS and acetate.

The compositions of the present invention may further comprise a preservative. Such preservatives include phenol, m-cresol and methylparaben. The most preferred preservative is methylparaben.

The compositions of the present invention may further comprise an isotonicity agent. Preferred isotonicity agents include glycerol and sodium chloride, with sodium chloride most preferred.

The composition of the present invention may further comprise additional pharmaceutically acceptable excipients designed for various purposes, such as maintaining formulation stability, maintaining particle resuspendability, preventing particle clumping, and the like. Such excipients are known to one skilled in the art or may be determined experimentally and are described in

references such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, PA, USA (1985) and Handbook of Pharmaceutical Excipients, 2nd Edition, American Pharmaceutical Association, Washington, D.C., USA (1995).

The compositions of the present invention are used to treat patients who have diabetes or hyperglycemia. The formulations of the present invention will typically provide derivatized protein at concentrations of from about 1 mg/mL to about 10 mg/mL. Present formulations of insulin products are typically characterized in terms of the concentration of units of insulin activity (units/mL), such as U40, U50, U100, and so on, which correspond roughly to about 1.4, 1.75, and 3.5 mg/mL preparations, respectively. The dose, route of administration, and the number of administrations per day will be determined by a physician considering such factors as the therapeutic objectives, the nature and cause of the patient's disease, the patient's gender and weight, level of exercise, eating habits, the method of administration, and other factors known to the skilled physician. In broad range, a daily dose would be in the range of from about 1 nmol/kg body weight to about 6 nmol/kg body weight (6 nmol is considered equivalent to about 1 unit of insulin activity). A dose of between about 2 and about 3 nmol/kg is typical of present insulin therapy.

The physician of ordinary skill in treating diabetes will be able to select the therapeutically most advantageous means to administer the formulations of the present invention. Parenteral routes of administration are preferred. Typical routes of parenteral administration of suspension formulations of insulin are the subcutaneous and intramuscular routes. The compositions and formulations of the present invention may also be administered by nasal, buccal, pulmonary, or occular routes. The compositions of the present invention are considered particularly advantageous for pulmonary delivery.

Methylparaben is the preferred preservative in formulations of the present invention.

Insulin or insulin analogs used to prepare derivatized proteins can be prepared by any of a variety of recognized peptide synthesis techniques including classical (solution) methods, solid phase methods, semi-synthetic methods, and more recent recombinant DNA methods. For example, see Chance, R. E., *et al.*, U.S. Patent No. 5,514,646, 7 May 1996; EPO publication number 383,472, 7 February 1996; Brange, J. J. V., *et al.* EPO publication number 214,826, 18 March 1987; and Belagaje, R. M., *et al.*, U.S. Patent No. 5,304,473, 19 April 1994, which disclose the preparation of various proinsulin and insulin analogs.

Generally, acylated insulins are prepared using methods known in the art. The publications listed above to describe derivatized proteins contain suitable methods to prepare derivatized proteins.

To prepare acylated proteins, the protein is reacted with an activated organic acid, such as an activated fatty acid. Activated fatty acids are derivatives of commonly employed acylating agents, and include activated esters of fatty acids, fatty acid halides, activated amides of fatty acids, such as, activated azolide derivatives [Hansen, L. B., WIPO Publication No. 98/02460, 22 January 1998], and fatty acid anhydrides. The use of activated esters, especially N-hydroxysuccinimide esters of fatty acids, is a particularly advantageous means of acylating a free amino acid with a fatty acid. Lapidot, *et al.* describe the preparation of N-hydroxysuccinimide esters and their use in the preparation of N-lauroyl-glycine, N-lauroyl-L-serine, and N-lauroyl-L-glutamic acid. The term "activated fatty acid ester" means a fatty acid which has been activated using general techniques known in the art [Riordan, J. F. and Vallee, B. L., Methods in Enzymology, XXV:494-499 (1972); Lapidot, Y., et al., J. Lipid Res. 8:142-145 (1967)]. Hydroxybenzotriazide (HOBT), N-hydroxysuccinimide

and derivatives thereof are particularly well known for forming activated acids for peptide synthesis.

To selectively acylate the ε-amino group, various protecting groups may be used to block the α-amino groups during the coupling. The selection of a suitable protecting group is known to one skilled in the art and includes *p*-methoxybenzoxycarbonyl (pmZ). Preferably, the ε-amino group is acylated in a one-step synthesis without the use of amino-protecting groups. A process for selective acylation at the Nε-amino group of Lys is disclosed and claimed by Baker, J. C., *et al.*, U.S. Patent No. 5,646,242, 8 July 1997. A process for preparing a dry powder of an acylated protein is disclosed and claimed by Baker, J. C., *et al.*, U.S. Patent No. 5,700,904, 23 December 1997.

An example of a process for preparing the precipitates and crystals of the present invention follows. A measured amount of the derivatized protein is dissolved in a volume of 0.1 N HCl. A separate solution is prepared by dissolving a measured amount of the protein in a volume of 0.1 N HCl. The two solutions are combined to form a mixture of the derivatized protein and protein. This mixture solution is stirred gently for about 5 to 10 minutes. To this solution is added a solution of zinc as one of its soluble salts, for example $Zn(II)Cl_2$, to provide from about 0.3 moles of zinc per mole of derivatized insulin to about 1.0 moles, or to as much as 2.0 moles, of zinc per mole of total protein (protein + derivatized protein). The resulting solution is stirred gently for about 5 to 10 minutes. To this solution is added an aqueous solution containing sodium chloride and sodium acetate whereupon a precipitate forms. The pH of this solution is adjusted to within the range 8 to 10 with gentle stirring, whereupon the precipitate dissolves to yield a clear solution. Optionally, the pH may then be adjusted to within the range 8 to 9. The solution is stirred gently for about 5 to 10

minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution is adjusted to about 5.5 with a small volume of 1 N HCl. Optionally, a small quantity (<1%) of ultralente "seed" crystals may be added. The resulting suspension is stirred gently to ensure homogeneity, then allowed to stand undisturbed at 25°C whereupon microcrystals are formed within a period from about 4 hours to about 10 days.

The microcrystals may then be formulated, for storage and administration to a patient, by combining the resulting preparation with an aqueous solution containing sodium chloride, sodium acetate, and zinc ions such that the final concentrations are approximately 0.08 mg/mL zinc ions, 1.6 mg/mL sodium acetate, 7 mg/mL sodium chloride, 1 mg/mL methylparaben, the final pH value is about 7.4, and the final total protein concentration is about 3.5 mg/mL.

Alternatively, the microcrystals may be separated from the mother liquor and introduced into a different solvent, for storage and administration to a patient. An example of an appropriate aqueous solvent is as follows: water for injection containing 1 mg/mL methylparaben, 0.08 mg/mL zinc ions, 1.6 mg/mL sodium acetate, 7 mg/mL sodium chloride, at a pH value of 7.4.

Another example of the way in which this invention may be practiced is described as follows. A solution is prepared containing about 14 mg/mL of acylated insulin, 7% sodium chloride, 0.1 M sodium acetate, and a quantity of zinc chloride adequate to give 0.3 to 0.9% of zinc ions by weight of the acylated insulin. The pH is adjusted to 5.5. Most of the acylated insulin then precipitates in the amorphous state which then converts to crystals upon standing at about 20°C. Upon completion of crystallization (approximately 4 hours to 10 days), a quantity of phenolic preservative calculated to give a preservative concentration appropriate to confer antimicrobial properties to the solution upon final dilution to the desired acylated insulin

concentration. Methylparaben is the preferred preservative. The pH of the formulation is adjusted to 7.4 with small quantities of sodium chloride and hydrochloric acid. The dilution step is performed to adjust the acylated insulin concentration to a desired value. Typically, 3.5 mg/mL is a preferred concentration.

Variations on the way in which the combination of ingredients is achieved are anticipated by the present invention and will be apparent to a person of ordinary skill. The crystallization step is an important aspect of the present invention and depends upon establishing appropriate conditions. Conditions considered important to this process are as follows: a total protein concentration of about 1 to 30 mg/mL and preferably from about 10 mg/mL to about 20 mg/mL and more preferably about 14 mg/mL; a zinc ion concentration of about 0.04 to about 0.2 mg/mL and preferably about 0.15 mg/mL; a sodium acetate concentration of about 4 to 12 mg/mL and preferably about 8 mg/mL; a sodium chloride concentration of about 40 to 100 mg/mL and preferably about 70 mg/mL; and a pH value of about 5.1 to 5.9 and preferably about 5.5.

In many of the preparations described below, the relative content of protein and derivatized protein of the crystals was estimated. To determine the amount of total protein, samples of re-dissolved precipitate or crystal, and of the supernatant above the precipitate or crystals, were analyzed by reversed-phase gradient HPLC, as described below.

Briefly, the analytical system relied on a C8 reversed-phase column, at 23°C. The flow rate was 1.0 mL/min and UV detection at 214 nm was used. Solvent A was 0.1% (vol:vol) trifluroacetic acid in 10:90 (vol:vol) acetonitrile:water. Solvent B was 0.1% (vol:vol) trifluroacetic acid in 90:10 (vol:vol) acetonitrile:water. The development program was (minutes, %B): (0.1,0);

(45.1,75); (50.1,100); (55,100); (57,0); (72,0). All changes were linear. Other analytical systems could be devised by the skilled person to achieve the same objective.

To prepare for the HPLC analysis, aliquots of the well-mixed suspensions were dissolved by diluting with either 0.01 N HCl or 0.03 N HCl. Results of HPLC analysis of these solutions permitted calculation of total protein. Aliquots of the suspensions were centrifuged for approximately 5 minutes in an Eppendorf 5415C microcentrifuge at 14,000 rpm. The decanted supernatant was diluted with either 0.01 N or 0.1 N HCl and analyzed by HPLC. The precipitate was washed by re-suspending in Dulbecco's phosphate buffered saline (without calcium or magnesium) and re-pelleted by centrifugation. The buffer was decanted and the solid was re-dissolved in 0.01 N HCl. The re-dissolved precipitate was analyzed by HPLC.

HPLC was used to confirm the presence of the expected proteins in the acidified suspension, re-dissolved precipitate, and supernatant and also to determine protein concentrations. The retention times of peaks in the chromatograms of the re-dissolved precipitates were compared with the retention times observed for the proteins and derivatized proteins used to make the formulations. The agreement between retention times was always good, showing that the proteins and derivatized proteins were actually incorporated into the microcrystals. Concentrations of protein and derivatized protein were determined by comparing the appropriate peak areas to the areas of a standard. A 0.22 mg/mL solution of derivatized insulin was used as the standard for the purpose of determining the retention time.

The present invention may be better understood with reference to descriptions of the following preparations. These example preparations are intended to be

representative of specific embodiments of the invention, and are not intended as limiting the scope of the invention.

Preparation 1

Ultralente-like co-crystals of B29-Nε-pentanoyl-human insulin and human insulin (1:1)

An acidic solution of B29-Nε-pentanoyl-human insulin was prepared by dissolving 16.5 mg of a dry powder of B29-Nε-pentanoyl-human insulin in 200 microliters of 0.1 N HCl. A separate solution was prepared by dissolving 15.0 mg of a dry powder of human insulin (as zinc crystals) in 100 microliters of 0.1 N HCl. The two solutions were combined to form a mixture of B29-Nε-pentanoyl-human insulin and human insulin. This mixture solution was stirred gently for about 5 to 10 minutes. To this solution was added 10 microliters of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 2 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 8.3 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.51 with a small quantity of 1 N HCl and 1 N NaOH. Ultralente seed crystals were prepared by placing 1 mL of U100 Humulin U in an ultrasonicating bath and sonicating it for about 10 minutes. A small quantity (10 microliters) of the ultralente seed crystals were added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a

controlled temperature of 25°C for 3 days whereupon microcrystals formed. HPLC analysis showed less than 17% of the total protein remained in the supernatant.

The microcrystals were separated from the mother liquor, then analyzed by HPLC. The HPLC analysis showed that the microcrystals contained 47% B29-Nε-pentanoyl-human insulin.

Preparation 2

Ultralente-like co-crystals of B29-Nε-pentanoyl-human insulin and human insulin (1:3)

An acidic solution of B29-Nε-pentanoyl-human insulin was prepared by dissolving 7.6 mg of B29-Nε-pentanoyl-human insulin in 200 microliters of 0.1 N HCl. A separate solution was prepared by dissolving 23.3 mg of a dry powder of human insulin (as zinc crystals) in 100 microliters of 0.1 N HCl. The two solutions were combined to form a mixture of B29-Nε-pentanoyl-human insulin and human insulin. This mixture solution was stirred gently for about 5 to 10 minutes. To this solution was added 10 microliters of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 2 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 8.3 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.36 with a small quantity of 1 N HCl and 1 N NaOH. Ultralente seed crystals were prepared by placing 1 mL of U100 Humulin

U in an ultrasonicating bath and sonicating it for about 10 minutes. A small quantity (10 microliters) of the ultralente seed crystals were added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed. HPLC analysis showed less than 10% of the total protein remained in the supernatant.

The microcrystals were separated from the mother liquor, then analyzed by HPLC. The HPLC analysis showed that the microcrystals contained 21% B29-Nε-pentanoyl-human insulin.

Preparation 3

Ultralente-like co-crystals of B29-Nε-pentanoyl-human insulin and human insulin (9:1)

An acidic solution of B29-Nε-pentanoyl-human insulin was prepared by dissolving 14.6 mg of a dry powder of B29-Nε-pentanoyl-human insulin in 200 microliters of 0.1 N HCl. A separate solution was prepared by dissolving 2.3 mg of a dry powder of human insulin (as zinc crystals) in 50 microliters of 0.1 N HCl. The two solutions were combined to form a mixture of B29-Nε-pentanoyl-human insulin and human insulin. This mixture solution was stirred gently for about 5 to 10 minutes. To this solution was added 10 microliters of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 1 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 8.1 with a small quantity of 1 N HCl. The

solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.45 with a small quantity of 1 N HCl and 1 N NaOH. Ultralente seed crystals were prepared by placing 1 mL of U100 Humulin U in an ultrasonicating bath and sonicating it for about 10 minutes. A small quantity (10 microliters) of the ultralente seed crystals were added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed. HPLC analysis showed less than 4% of the total protein remained in the supernatant.

The microcrystals were separated from the mother liquor, then analyzed by HPLC. The HPLC analysis showed that the microcrystals contained 85% B29-Nε-pentanoyl-human insulin.

Preparation 4

Ultralente-like co-crystals of B29-Nε-octanoyl-human insulin and human insulin (1:4)

An acidic solution of B29-Nε-octanoyl-human insulin was prepared by dissolving 7.6 mg of a dry powder of B29-Nε-octanoyl-human insulin in 200 microliters of 0.1 N HCl. A separate solution was prepared by dissolving 24.9 mg of a dry powder of human insulin (as zinc crystals) in 100 microliters of 0.1 N HCl. The two solutions were combined to form a mixture of B29-Nε-octanoyl-human insulin and human insulin. This mixture solution was stirred gently for about 5 to 10 minutes. To this solution was added 10 microliters of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 1 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a

precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 8.6 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.51 with a small quantity of 1 N HCl and 1 N NaOH. Ultralente seed crystals were prepared by placing 1 mL of U100 Humulin U in an ultrasonicating bath and sonicating it for about 10 minutes. A small quantity (10 microliters) of the ultralente seed crystals were added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed. HPLC analysis showed less than 2% of the total protein remain in the supernatant.

The microcrystals were separated from the mother liquor, then analyzed by HPLC. The HPLC analysis showed that the microcrystals contained 18% B29-Nε-octanoyl-human insulin.

Preparation 5

Ultralente-like co-crystals of B29-Nε-octanoyl-human insulin and human insulin (1:2)

An acidic solution of B29-Nε-octanoyl-human insulin was prepared by dissolving 12.2 mg of a dry powder of B29-Nε-octanoyl-human insulin in 200 microliters of 0.1 N HCl. A separate solution was prepared by dissolving 20.0 mg of a dry powder of human insulin (as zinc crystals) in 100 microliters of 0.1 N HCl. The two solutions were combined to form a mixture of B29-Nε-octanoyl-human insulin and human insulin. This mixture solution was stirred gently for about 5 to 10 minutes. To this solution was added 10 microliters

of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 2 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 8.4 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.51 with a small quantity of 1 N HCl and 1 N NaOH. Ultralente seed crystals were prepared by placing 1 mL of U100 Humulin U in an ultrasonicating bath and sonicating it for about 10 minutes. A small quantity (10 microliters) of the ultralente seed crystals were added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed. HPLC analysis showed less than 1% of the total protein remained in the supernatant.

The microcrystals were separated from the mother liquor, then analyzed by HPLC. The HPLC analysis showed that the microcrystals contained 36% B29-Nε-octanoyl-human insulin.

Preparation 6

Ultralente-like co-crystals of B29-Nε-decanoyl-human insulin and human insulin (1:5)

An acidic solution of B29-Nε-decanoyl-human insulin was prepared by dissolving 5.1 mg of a dry powder of B29-Nε-decanoyl-human insulin in 200 microliters of 0.1 N HCl. A separate solution was prepared by dissolving 26.7 mg

of a dry powder of human insulin (as zinc crystals) in 100 microliters of 0.1 N HCl. The two solutions were combined to form a mixture of B29-Nε-decanoyl-human insulin and human insulin. This mixture solution was stirred gently for about 5 to 10 minutes. To this solution was added 40 microliters of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 2 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 9.4 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.45 with a small quantity of 1 N HCl and 1 N NaOH. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed. HPLC analysis showed that less than 1% of the total protein remained in the supernatant.

The microcrystals were separated from the mother liquor, then analyzed by HPLC. The HPLC analysis showed that the microcrystals contained 17% B29-Nε-decanoyl-human insulin.

Preparation 7

Ultralente-like crystals of B29-Nε-butanoyl-human insulin

An acidic solution of B29-Nε-butanoyl-human insulin was prepared by dissolving 33.3 mg of a dry powder of B29-Nε-butanoyl-human insulin in 400 microliters of 0.1 N HCl. This solution was stirred gently for about 5 to 10 minutes. To this solution was added 40 microliters of a

solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 2 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 8.9 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.35 with a small quantity of 1 N HCl and 1 N NaOH. A 100 microliter volume of seed crystals (0.3 mg/mL human zinc insulin crystals of approximate size 3 microns containing 0.8 mg/mL methylparaben and 0.29 mg/mL citric acid in water) was added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed.

Preparation 8

Ultralente-like crystals of B29-Nε-pentanoyl-human insulin

An acidic solution of B29-Nε-pentanoyl-human insulin was prepared by dissolving 16.0 mg of a dry powder of B29-Nε-pentanoyl-human insulin in 200 microliters of 0.1 N HCl. This solution was stirred gently for about 5 to 10 minutes. To this solution was added 20 microliters of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 1 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a

precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 9.1 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a 0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.45 with a small quantity of 1 N HCl and 1 N NaOH. A 100 microliter volume of seed crystals (0.3 mg/mL human zinc insulin crystals of approximate size 3 microns containing 0.8 mg/mL methylparaben and 0.29 mg/mL citric acid in water) was added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed.

Preparation 9

Ultralente-like crystals of B29-Nε-hexanoyl-human insulin

An acidic solution of B29-Nε-hexanoyl-human insulin was prepared by dissolving 16.7 mg of a dry powder of B29-Nε-hexanoyl-human insulin in 200 microliters of 0.1 N HCl. This solution was stirred gently for about 5 to 10 minutes. To this solution was added 20 microliters of a solution of 0.15 M zinc chloride (prepared by dissolving zinc oxide in HCl). The resulting solution was stirred gently for about 5 to 10 minutes. To this solution was added 1 mL of an aqueous solution containing 70 mg/mL sodium chloride and 13.6 mg/mL sodium acetate whereupon a precipitate formed. The pH of this solution was adjusted to within the range 8 to 10 with a small quantity of 1 N NaOH, whereupon, with gentle stirring, the precipitate dissolved to yield a clear solution. The pH was then adjusted to 9.6 with a small quantity of 1 N HCl. The solution was then stirred gently for about 5 minutes then filtered through a

0.22 micron, low-protein binding filter. The pH of the resulting solution was then adjusted to 5.49 with a small quantity of 1 N HCl and 1 N NaOH. A 100 microliter volume of seed crystals (0.3 mg/mL human zinc insulin crystals of approximate size 3 microns containing 0.8 mg/mL methyl paragon and 0.29 mg/mL citric acid in water) was added and the resulting suspension was stirred gently to ensure uniformity. The resulting preparation was allowed to stand undisturbed at a controlled temperature of 25°C for 3 days whereupon microcrystals formed.

I claim:

1. Ultralente-like crystals, comprising:

a) a derivatized protein selected from the group consisting of the human insulin derivatives formed by derivatizing human insulin with the saturated, straight-chain fatty acids having from 4 to 16 carbon atoms such that the fatty acids form amide bonds with the ε-amino group of the B29-lysine of human insulin; and

b) a divalent metal cation.

2. The crystals of Claim 1, wherein the human insulin derivative is selected from the group consisting of B29-butanoyl-human insulin, B29-pentanoyl-human insulin, and B29-hexanoyl-human insulin.

3. An insoluble composition, comprising the crystals of any one of Claims 1-2.

4. The insoluble composition of Claim 3, further comprising amorphous precipitate.

5. Ultralente-like crystals, comprising:

a) a protein selected from the group consisting of insulin and insulin analogs;

b) a derivatized protein selected from the group consisting of the human insulin derivatives formed by derivatizing human insulin with the saturated, straight-chain fatty acids having from 4 to 16 carbon atoms such that the fatty acids form amide bonds with the ε-amino group of the B29-lysine of human insulin; and

c) a divalent metal cation.

6. The crystals of Claim 3, wherein the protein is human insulin.

7. The crystals of Claim 3, wherein the protein is a monomeric insulin analog.

8. The crystals of Claim 5, wherein the protein is LysB28,ProB29-human insulin analog.

9. The crystals of any one of Claims 3-6, wherein the molar proportion of derivatized protein is from 15% to 90% of the total protein.

10. The crystals of any one of Claims 1-9, wherein the divalent metal cation is zinc, which is present at about 0.3 mole per mole of total protein to about 2 moles per mole of total protein.

11. An insoluble composition, comprising the crystals of any one of Claims 3-8.

12. The insoluble composition of Claim 11, further comprising amorphous precipitate.

13. A pharmaceutical composition, comprising an insoluble phase and a solution phase, wherein the insoluble phase is comprised of the insoluble composition of Claim 3, Claim 4, Claim 11, or Claim 12, and wherein the soluble phase is comprised of an aqueous solvent.

14. The pharmaceutical composition of Claim 13 wherein the solution phase is further comprised of a preservative at a concentration of about 0.5 mg per mL to about 6 mg per mL of solution, a pharmaceutically acceptable buffer, and an isotonicity agent.

15. A method of treating diabetes comprising administering the crystals of any one of Claims 1-2 or Claims 5-10 to a patient in need thereof in a quantity sufficient to regulate blood glucose levels in the patient.

16. A method of treating diabetes comprising administering the insoluble compositions of Claim 13 or Claim 14 to a patient in need thereof in a quantity sufficient to regulate blood glucose levels in the patient.

17. A method of treating hyperglycemia comprising administering the crystals of any one of Claims 1-2 or Claims 5-10 to a patient in need thereof in a quantity sufficient to regulate blood glucose levels in the patient.

18. A method of treating hyperglycemia comprising administering the insoluble compositions of any one of Claim 13 or Claim 14 to a patient in need thereof in a quantity sufficient to regulate blood glucose levels in the patient.

19. A process for preparing the crystals of Claim 1 or Claim 2, comprising:

a) preparing a crystallization solution comprising the derivatized protein, a buffer, a salt, and a divalent cation; and

b) allowing time for crystallization to occur.

20. A process for preparing the crystals of any one of Claims 5-10, comprising:

a) preparing a crystallization solution comprising a protein, a derivatized protein, a buffer, a salt, and a divalent cation;

b) combining the crystallization solution of a) with a nucleating seed suspension; and

c) allowing time for crystallization to occur.

INTERNATIONAL SEARCH REPORT

Int. Ional Application No
PCT/US 00/15037

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/62 A61K38/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 42367 A (HANSEN PHILIP ;JENSEN STEEN (DK); NOVONORDISK AS (DK)) 1 October 1998 (1998-10-01) page 3, line 19 - line 24 page 3, line 27 - line 28 page 4, line 5 - line 29 page 4, line 13 - line 20 page 8, line 4 - line 7; claims	1-19
X	WO 98 42368 A (HANSEN PHILIP ;JENSEN STEEN (DK); NOVONORDISK AS (DK)) 1 October 1998 (1998-10-01) page 3, line 29 -page 4, line 26 page 5, line 15 - line 33; claims	1-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2000	04/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/US 00/15037

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.L. WHITTINGHAM ET AL.: "Crystal Structure of a Prolonged-Acting Insulin with albumin-Binding Properties " BIOCHEMISTRY, vol. 36, 11 March 1997 (1997-03-11), pages 2826-2831, XP002147629 EASTON, PA US page 2827, left-hand column, paragraph 2 page 2830, left-hand column, paragraph 2 -page 2831, left-hand column, last paragraph	1-19
A	EP 0 646 379 A (LILLY CO ELI) 5 April 1995 (1995-04-05) claims; examples	1-19

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-19 relate to a product and composition defined by reference to a desirable characteristic or property, namely being "ultralente-like".
The claims cover all products and compositions having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products and compositions. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product and compositions by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to insulin-zinc crystals as such and in particular to those crystals which can be administered pulmonary.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 00/15037

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